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## Differential effects of triterpene glycosides, frondoside A and cucumarioside A<sub>2</sub>-2 isolated from sea cucumbers on caspase activation and apoptosis of human leukemia cells

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### ABSTRACT

**Frondoside A is a pentaoside having an acetyl moiety at the aglycon ring and xylose as a third monosaccharide residue. Cucumarioside A<sub>2</sub>-2 is a pentaoside having glucose as a third monosaccharide unit. We compared the effects of frondoside A and A<sub>2</sub>-2 for cell death-inducing capability with close attention paid to structure–activity relationships. Both frondoside A and A<sub>2</sub>-2 strongly induced apoptosis of leukemic cells. Frondoside A-induced apoptosis was more potent and rapid than A<sub>2</sub>-2-induced apoptosis. A<sub>2</sub>-2-induced but not frondoside A-induced apoptosis was caspase-dependent. This suggests that holothurians may induce apoptosis of leukemic cells caspase-dependently or -independently, depending on the holothurian structure.**

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### 1. Introduction

Apoptosis is characterized by specific morphological and biochemical changes with activation of the cysteine aspartyl proteases family caspases [1]. This family includes two subclasses of initiator and effector enzymes [2]. However, there is growing evidence that apoptotic pathways also can be activated without participation of caspases, through mechanisms involving activation of other classes of proteases such as calpains (calcium-dependent proteases), cathepsins, serine proteases, granzymes, etc. [3]. These proteases often cooperate with caspases, but they also can provoke characteristic apoptotic changes in a caspase-independent manner [4].

Glycosides, substances consisting of a sugar moiety (carbohydrate chain) and triterpene or steroid aglycon are widely distrib-

uted in plants and have attracted the most attention. Triterpene oligoglycosides were found in marine invertebrates belonging to the class *Holothurioidea* in the animal kingdom and in some sponges [5]. However, only a few papers regarding the structure and biological activity of the holothurians have been published until now.

Frondoside A, a major triterpene glycoside isolated from sea cucumber *Cucumaria frondosa*, has a sulfate, acetoxyl group at C-16 of the aglycon, pentasaccharide chain, xylose at the third monosaccharide residue, and 3-O-methylglucose as terminal monosaccharide residue [6]. Cucumarioside A<sub>4</sub>-2, a glycoside isolated from *Cucumaria japonica*, has monosulfated pentaoside having 16-keto group in aglycon and glucose residue as a third monosaccharide unit in the carbohydrate chain [7]. The substance A<sub>2</sub>-2 is probably biogenetically connected with A<sub>4</sub>-2 and has 3-O-methylglucose instead of glucose as the terminal monosaccharide unit [7]. The main structural difference between frondoside A and A<sub>2</sub>-2 is in the functional group at C-16 of the aglycon (acetoxyl or keto group) and the third carbohydrate unit in the carbohydrate chain. In this study, we observed differences in the potency of the cytotoxicity and apoptotic pathways activated by frondoside A and A<sub>2</sub>-2.

**Abbreviations:** PARP, poly (ADP ribose) polymerase; ER, endoplasmic reticulum; DiOC<sub>6</sub>(3), 3,3'-dihexyloxycarbocyanine iodide; AIF, apoptosis-inducing factor

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## 2. Materials and methods

### 2.1. Reagents

The chemical structures of frondoside A, A<sub>2</sub>-2, and A<sub>4</sub>-2 are shown in Fig. 1. The frondoside A and cucumariosides were extracted and purified as previously described [8,9]. Annexin-V was from BD Biosciences Clontech (Palo Alto, CA, USA). Anti-poly (ADP ribose) polymerase (PARP) and cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against procaspases were purchased from Cell Signaling Technology (Beverly, MA, USA). The inhibitors zDEVD-fmk, zVAD-fmk, and zIETD-fmk were purchased from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Cell culture

HL-60, NB4, and THP-1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and with antibiotic–antimycotic cocktail and were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 2.3. Neutral red release assay

Cytotoxicity was studied using the neutral red release assay according to Borenfreund and Puerner [8]. The neutral red re-

lease assay is an in vitro viability test, based on the incorporation of neutral red stain into the lysosomes of viable cells. Briefly,  $1 \times 10^4$  exponentially growing cells were seeded in 96-well plates with various concentrations of frondoside A, A<sub>2</sub>-2, or A<sub>4</sub>-2 for 24 h. Cells were washed with 200  $\mu$ l PBS and filtered neutral red stock solution (4%, w/v, 0.9% NaCl) was diluted 1:60 in serum-free media and added to each well. After 4 h incubation, cells were washed twice and 150  $\mu$ l of lysing solution was added (50%, v/v, ethanol with 1%, v/v, acetic acid) to the wells. Absorbance at 540 nm was measured using a spectrophotometric microplate reader. All results were expressed as percentage of viable cells.

### 2.4. Apoptosis assay

Leukemia cells were treated with different concentrations of frondoside A, A<sub>2</sub>-2, or A<sub>4</sub>-2 for 6 h. After incubation, cells were harvested, washed with phosphate buffered saline (PBS, pH 7.4), centrifuged, and stained with annexin V-fluorescein-isothiocyanate (FITC) and propidium iodide (PI) in 100  $\mu$ l of binding buffer (10 mM HEPES, pH 7.4/140 mM NaCl/2.5 mM CaCl<sub>2</sub>) for 15 min in the dark. The samples were analyzed by flow cytometry using a FACScan flow cytometer, which differentiated early apoptotic cells (annexin V-FITC positive), late apoptotic and/or necrotic cells (annexin V-FITC and PI positive), and viable cells (unstained). The apoptotic cells were determined as annexin V+/PI– and annexin V+/PI+ cells.

### 2.5. Western blot analysis

HL-60 cells were harvested, washed with ice-cold PBS, and treated with lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10% glycerol, 1% Triton X-100 accompanied by protease inhibitors. Proteins were separated by 10% or 18% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with a blocking buffer (10 mM Tris–HCl, 0.15 M NaCl, 0.1% NaN<sub>3</sub>, and 5% skim milk) for 1 h at 25 °C and incubated with primary polyclonal antibodies directed against procaspase-3, -8, -7, -9, and -12, the cleavage form of caspases-3, PARP, cytochrome c, and  $\beta$ -actin in blocking buffer overnight at 4 °C. The membranes were incubated with the secondary antibodies for 1 h at 25 °C. The signals were detected using ECL chemiluminescence following the manufacturer's instructions.

### 2.6. Mitochondrial membrane permeability assay

HL-60 cells ( $1 \times 10^6$ /ml) were incubated with 40 nM of 3,3'-dihexyloxycarbocyanine iodide [DiOC<sub>6</sub>(3), Molecular probes] for 30 min at 37 °C and then washed with PBS. Stained cells were analyzed by flow cytometry.

### 2.7. Detection of cytochrome c in cytosolic fraction

Cytosolic and mitochondrial proteins were separated as described previously [9]. In brief, cells treated with frondoside A, A<sub>2</sub>-2, or etoposide for 6 h were collected and resuspended in mitochondrial isolation buffer (20 mM HEPES–KOH, pH 7.5, 210 mM sucrose, 70 mM mannitol, 1 mM EDTA, 1 mM DTT, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl) and protease inhibitor cocktail (Boehringer Mannheim) supplemented with 10  $\mu$ M digitonin. Suspensions were incubated at 37 °C for 5 min and centrifuged at 12000 $\times$ g for 15 min. The supernatant (cytosolic fraction) was collected for western blotting.

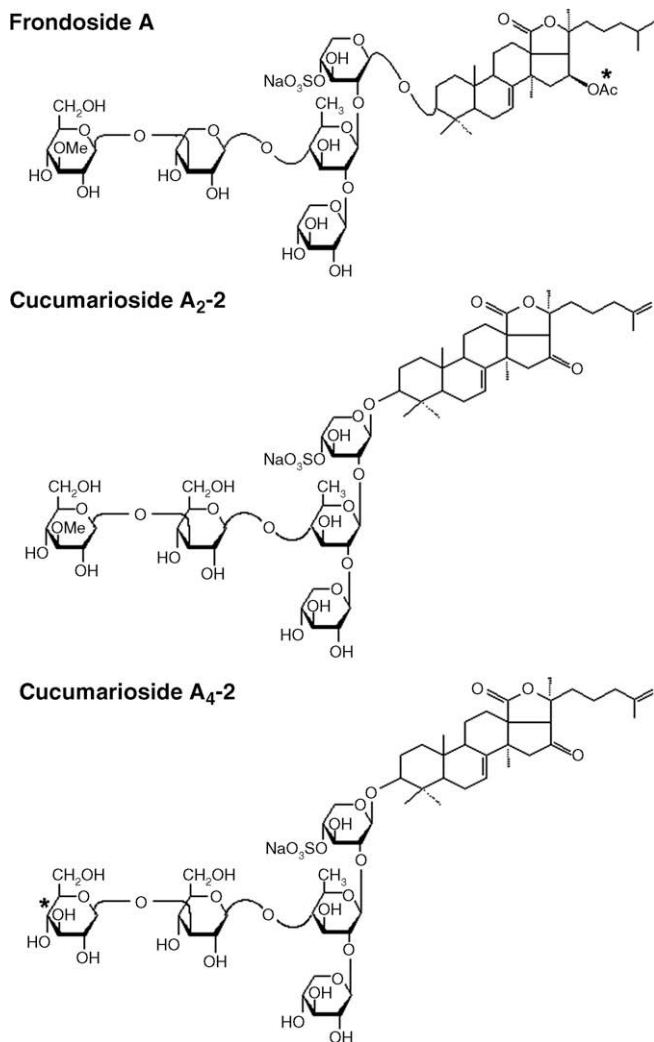


Fig. 1. Chemical structure of triterpene glycoside frondoside A, A<sub>2</sub>-2, and A<sub>4</sub>-2.

## 2.8. Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (S.D.). Statistical analyses were carried out using Student's *t*-tests. Time-course and dose-response were analyzed by one-way analysis of variance (ANOVA) with the post-test. A *P* value of  $<0.05$  was considered significant.

## 3. Results

### 3.1. Cytotoxic effects of frondoside A and cucumariosides

To explore the effects of frondoside A, A<sub>2</sub>-2, and A<sub>4</sub>-2 on leukemic cell viability, HL-60 cells were cultured with different concentrations of these agents for 24 h and cytotoxicity was measured by neutral red release assay. As shown in Fig. 2A, frondoside A had a dose-dependent influence on HL-60 viability and totally inhibited cell viability at concentrations at or above 2  $\mu$ M (ANOVA,  $F = 2206$ ,  $P < 0.001$ ). A<sub>2</sub>-2 and A<sub>4</sub>-2 also had a cytotoxic effect (ANOVA, A<sub>2</sub>-2;  $F = 3153$ ,  $P < 0.001$ , A<sub>4</sub>-2;  $F = 2511$ ,  $P < 0.001$ ) but it was less than that for frondoside A. It also showed that frondoside A

treatment of HL-60 cells increased the sub-G1 fraction (data not shown). Treatment with 5  $\mu$ M A<sub>2</sub>-2 or A<sub>4</sub>-2 increased the sub-G1 fraction as much as 1  $\mu$ M frondoside A treatment.

### 3.2. Apoptotic effect of frondoside A and cucumariosides on leukemia cells

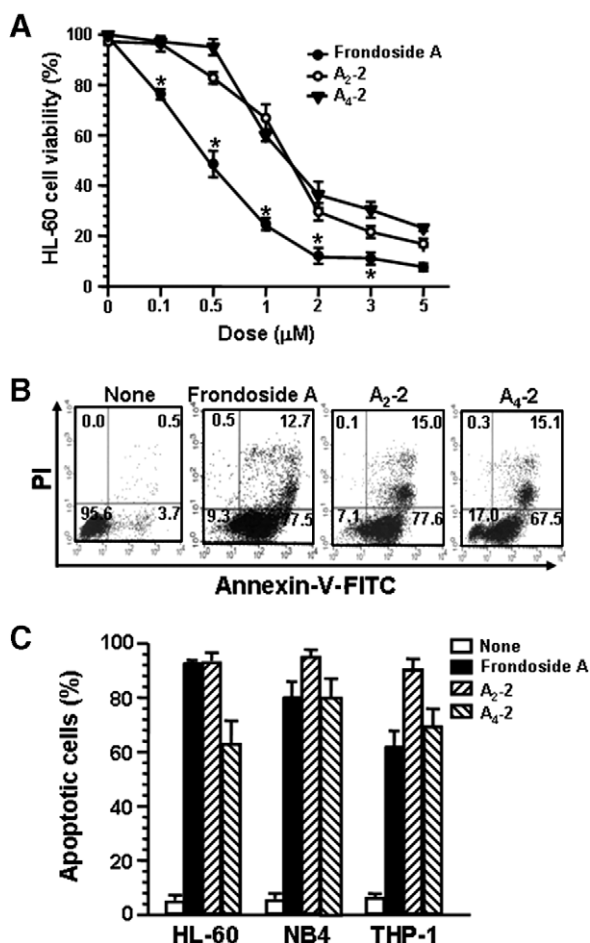
The apoptosis rate was determined by measuring the extent of annexin V-FITC binding. The apoptotic rates of HL-60 cells were increased after 6 h of culture by 1  $\mu$ M of frondoside A, 5  $\mu$ M of A<sub>2</sub>-2, and 5  $\mu$ M of A<sub>4</sub>-2 (Fig. 2B). Four different leukemia cell lines were tested for the ability of frondoside A to induce apoptosis. HL-60, NB4, THP-1, and K562 cells were treated with 1  $\mu$ M frondoside A for 6 h (Fig. 2C). Frondoside A had a stronger apoptotic effect on HL-60 and NB4 cells than on THP-1 cells. The apoptosis of other leukemic cells was also induced by A<sub>2</sub>-2 and A<sub>4</sub>-2, although higher concentrations were needed than that of frondoside A to induce a degree of apoptosis similar to frondoside A.

The frondoside A-provoked apoptosis in HL-60 cells and THP-1 cells in a dose-dependent manner (0.5–5  $\mu$ M for 6 h, Fig. 3A). Frondoside A-induced a rapid increase in the proportion of apoptotic cells (up to 90.2% at 1  $\mu$ M concentration for HL-60 cells) while the apoptotic rate in THP-1 cells at the same concentration was  $\sim 60\%$  (ANOVA, HL-60 cells;  $F = 218.2$ ,  $P < 0.001$ , THP-1 cells;  $F = 80.0$ ,  $P < 0.001$ ). At the same concentrations, the A<sub>2</sub>-2 and A<sub>4</sub>-2 induced respectively,  $15 \pm 5\%$  and  $42 \pm 7\%$  cell death in HL-60 cells. At 5  $\mu$ M, A<sub>2</sub>-2 and A<sub>4</sub>-2-induced apoptosis was evident after 6 h treatment of HL-60 cells (ANOVA, A<sub>2</sub>-2;  $F = 293.2$ ,  $P < 0.001$ , A<sub>4</sub>-2;  $F = 161.2$ ,  $P < 0.001$ ).

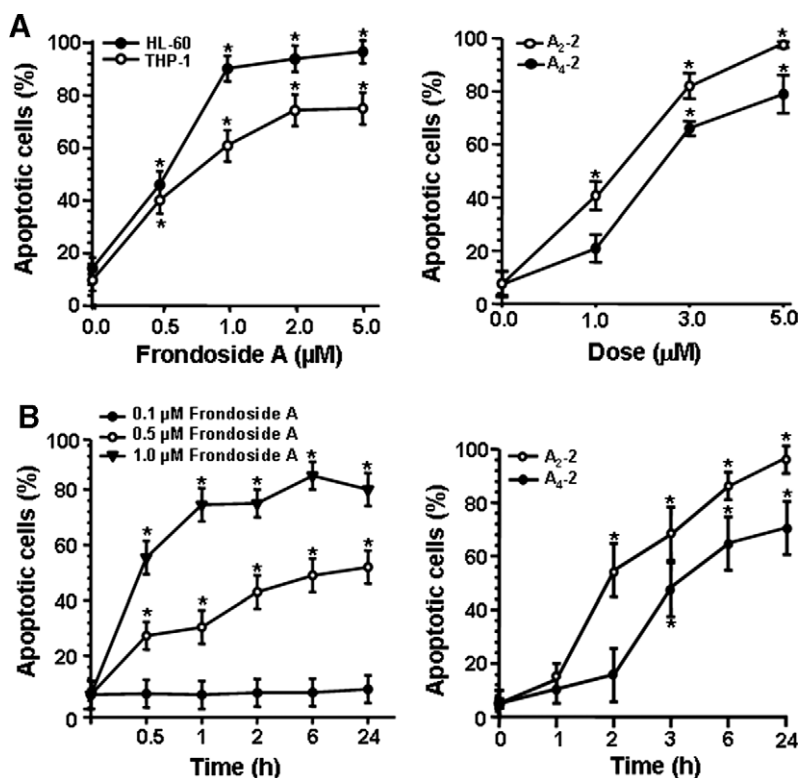
Next, we tested the time-dependence of the effect of frondoside A at different concentrations on HL-60 cells (Fig. 3B). At low concentrations (0.1  $\mu$ M), frondoside A-induced apoptosis in only 10% of cells and the amount did not change after 24 h of incubation. At 0.5  $\mu$ M concentration (0.5–1 h incubation), frondoside A significantly increased the proportion of cells in apoptosis and for 24 h incubation slightly increased the proportion of cells in apoptosis to 50% (ANOVA,  $F = 34.6$ ,  $P < 0.001$ ). At high concentrations (1  $\mu$ M), frondoside A elicited apoptosis in  $\sim 50\%$  of cells as early as 30 min after incubation and this increased to 75% after 6 h incubation (ANOVA,  $F = 179.0$ ,  $P < 0.001$ ). At 2 h after treatment, A<sub>2</sub>-2 (3  $\mu$ M) had induced 60–80% of HL-60 cells to undergo apoptosis (ANOVA multiple comparison,  $P < 0.001$ ), while A<sub>4</sub>-2 at the same concentration induced only 15% of cells to undergo apoptosis (ANOVA multiple comparison,  $P = 0.494$ ). Significant increases in cell death were also observed at 6 h after A<sub>2</sub>-2 or A<sub>4</sub>-2 treatment. Although A<sub>4</sub>-2 was less active than A<sub>2</sub>-2, it showed similar apoptosis-inducing activity in HL-60 cells. These results indicate that frondoside A is more toxic to leukemic cells than cucumariosides.

### 3.3. Effects of frondoside A on intracellular levels of apoptotic proteins in HL-60 cells

We measured the staining of cells using DiOC<sub>6</sub>(3), which reflects changes in mitochondrial membrane potential. As shown in Fig. 4A, a mitochondrial membrane permeability assay did not show significant changes in DiOC<sub>6</sub>(3) staining of HL-60 cells at 6 h after treatment with frondoside A and cucumariosides. In addition, the accumulation of cytochrome c in the cytosolic fraction isolated from frondoside A-, A<sub>2</sub>-2-, or A<sub>4</sub>-2-treated HL-60 cells was not observed, whereas cells treated with etoposide showed a marked increase in the presence of cytochrome c in the cytosolic fraction. Next, we compared time courses of apoptosis and activation of procaspases in frondoside A-treated HL-60 cells. The levels of all tested procaspase-3, -8, and -9 proteins in lysates from frondoside A-treated (for up to 2 h) HL-60 cells were not changed, whereas, at these time points, frondoside A-induced apoptosis in



**Fig. 2.** Effects of frondoside A and cucumariosides on cytotoxicity and apoptosis in leukemic cells. (A) HL-60 cells were treated with different concentrations of the frondoside A, A<sub>2</sub>-2, or A<sub>4</sub>-2 for 24 h. Neutral red solution was added to the washed cells. After 4 h incubation, the viability of cells was detected by measuring absorbance at 540 nm. (B) HL-60 cells were treated with frondoside A (1  $\mu$ M), A<sub>2</sub>-2 (5  $\mu$ M), or A<sub>4</sub>-2 (5  $\mu$ M) for 6 h. The cells were then subject to annexin V-FITC and PI staining and apoptosis was measured by flow cytometry. Representative experiments are shown. (C) Leukemic cells were treated with or without frondoside A, A<sub>2</sub>-2, or A<sub>4</sub>-2 as shown in panel B. The results represent the mean  $\pm$  S.D. of three independent experiments. \* $P < 0.01$  compared to A<sub>2</sub>-2 (Student's *t*-test).



**Fig. 3.** Dose- and time-dependent apoptotic effects of frondoside A and cucumariosides on leukemic cells. (A) In the left panel, HL-60 cells and THP-1 cells were treated with the indicated concentrations of the frondoside A for 6 h. In the right panel, HL-60 cells were treated with the indicated concentrations of the A<sub>2</sub>-2 or A<sub>4</sub>-2 for 6 h. (B) In the left panel, HL-60 cells were treated with different concentrations of the frondoside A for the indicated times. In the right panel, HL-60 cells were treated with 3 μM of A<sub>2</sub>-2 (ANOVA,  $F = 204.6$ ,  $P < 0.001$ ) or A<sub>4</sub>-2 (ANOVA,  $F = 133.4$ ,  $P < 0.001$ ) for the indicated times. \* $P < 0.001$  compared to untreated control (ANOVA).

50–70% of cells (Fig. 4B). After 6 h incubation of HL-60 cells with frondoside A, however, the levels of procaspases-8 and -3 significantly decreased. Moreover, the time-dependent changes in the levels of cleaved caspase-3 and PARP, a typical substrate for caspase-3, were closely related to the changes in procaspase-3. These results suggest that frondoside A initiates apoptosis in a caspase-independent manner. We also determined whether caspase-7, which is also activated in response to endoplasmic reticulum (ER) stress, is also activated in response to frondoside A. The change in cleavage of procaspase-7 in the frondoside A-treated HL-60 cells was similar to that of caspase-3 in time-dependent experiments. In comparison, western blot analysis of HL-60 cells treated with A<sub>2</sub>-2 or A<sub>4</sub>-2 showed that the cleavages of procaspase-3 and PARP but not procaspases-8, -9, and -12 were significantly increased after 6 h treatment (Fig. 4C).

#### 3.4. Effects of caspase inhibitors on frondoside A-induced apoptosis in HL-60 cells

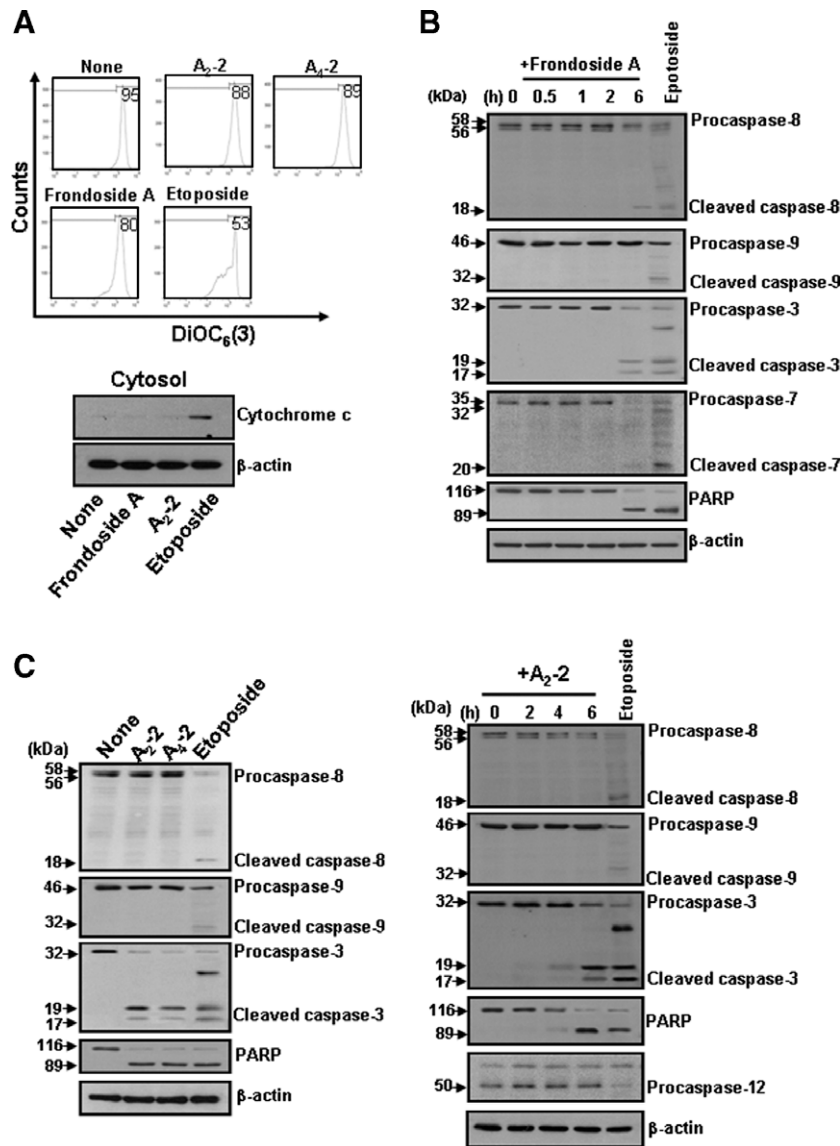
HL-60 cells were treated with a high concentration of zDEVD-fmk (200 μM), a caspase-3 inhibitor, alone or in combination with frondoside A for 6 h. Depending on the time point and the frondoside A concentration used, inhibition of cell death with zDEVD-fmk varied between 0% and 10%, indicating that cell death is largely caspase-independent (Fig. 5A). zVAD-fmk (200 μM) also did not show significant inhibition of the apoptosis induced by frondoside A. As positive controls, zDEVD-fmk (200 μM) or zVAD-fmk (200 μM) each prevented the apoptosis of HL-60 cells induced by etoposide, reducing the level of apoptotic cells from 70% to 15% and 10%, respectively. In comparison, when HL-60 cells were pre-incubated with a broad spectrum inhibitor of caspase activity followed by a 6 h exposure to A<sub>2</sub>-2 or A<sub>4</sub>-2 (3–5 μM), zVAD-fmk

blocked A<sub>2</sub>-2- or A<sub>4</sub>-2-induced apoptosis. A similar result was obtained when zDEVD-fmk was used, although the magnitude of the blocking effect by zDEVD-fmk was less than that by zVAD-fmk (Fig. 5B). In contrast, z-IETD-fmk (a caspase-8 inhibitor) was only marginally effective in preventing apoptosis of HL-60 cells induced by A<sub>2</sub>-2 or A<sub>4</sub>-2. Next, we investigated whether caspase inhibitors block activation of caspases by western blot because frondoside A and A<sub>2</sub>-2 reduced procaspase-3, and increased cleaved caspase-3 and PARP cleavage (Fig. 4B and C). As shown in Fig. 5C, decrease of procaspase-3, increase of cleaved caspase-3, and cleavage of PARP induced by A<sub>2</sub>-2 or frondoside A, were efficiently blocked by either zDEVD-fmk (200 μM) or zVAD-fmk (200 μM). Moreover, frondoside A-induced cleavage of procaspase-8 was blocked by zVAD-fmk. These results support caspase-independent activation of apoptosis by frondoside A and the caspase-3-dependent activation of apoptosis by cucumariosides.

#### 4. Discussion

On the basis of our previous observations regarding the importance of the structure of the aglycon and carbohydrates in conferring membranolytic activity [10], we compared the cytotoxicity of two holoturians against various leukemia cells. The LD<sub>50</sub> of frondoside A and cucumarioside for mice was 9.9 mg/kg and 10 mg/kg, respectively after intraperitoneal injection [7,11]. This suggests that acute toxicity for these two compounds is similar. On the other hand, our unpublished study showed that hemolytic activity of frondoside A was three times more potent than A<sub>2</sub>-2, which is consistent with the data on anti-tumoral effects obtained from our present study. The LD<sub>50</sub> of frondoside A in HL-60 cells was in the low or submicromolar range and was approximately 5–10 fold lower than LD<sub>50</sub>s for cucumariosides A<sub>2</sub>-2 and A<sub>4</sub>-2.



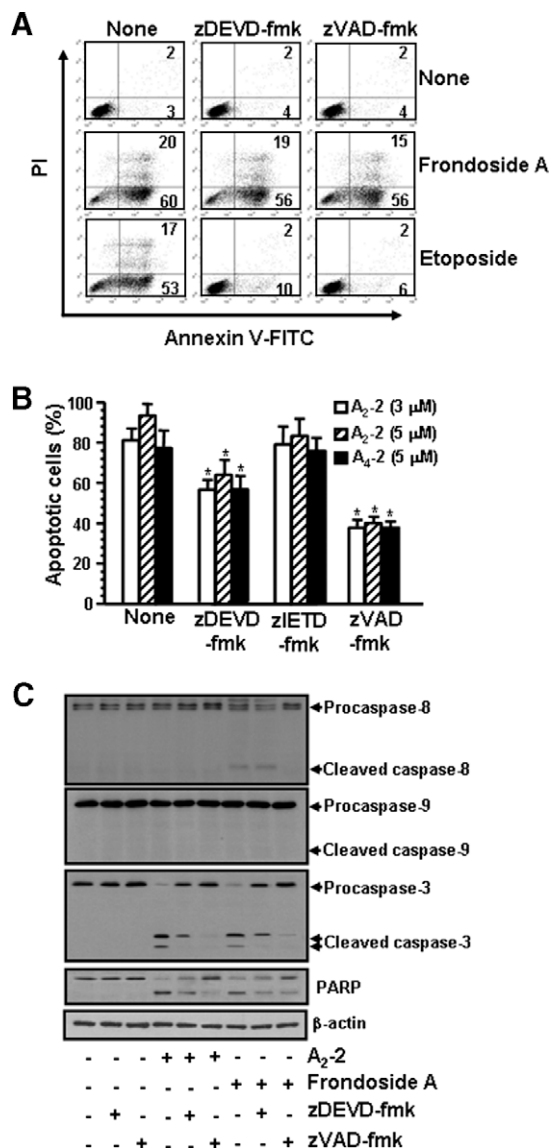


**Fig. 4.** Caspase-independent apoptosis in frondoside A-treated leukemic cells. (A) HL-60 cells were treated with frondoside A (1 μM), A<sub>2</sub>-2 (3 μM), A<sub>4</sub>-2 (3 μM), or etoposide (10 μM) for 6 h. In the upper panel, cells were incubated with 40 nM DiOC<sub>6</sub>(3) for 30 min and subjected to flow cytometry. In the lower panel, the levels of cytochrome c (12.5 kDa) in the cytosolic fraction were measured by western blot. As a positive control, 10 μM etoposide was used. Actin was used as loading control for the cytosolic fraction. (B) HL-60 cells were treated for the indicated times with frondoside A (1 μM) or etoposide (10 μM) for 6 h. (C) In the left panel, HL-60 cells were treated with A<sub>2</sub>-2 (3 μM), A<sub>4</sub>-2 (3 μM), or etoposide for 6 h. In the right panel, cells were treated with A<sub>2</sub>-2 (3 μM) for the indicated times or etoposide for 6 h. The expression of caspase-associated proteins was detected by western blot analysis. The expression of β-actin protein served as a loading control.

The structures of both the aglycon part and the carbohydrate chain are very important for anti-tumor activity. It has been reported that the presence of acetyl groups usually increases cytotoxic potency [12]. Similarly, monosulfated cucumariosides A<sub>2</sub>-2 and A<sub>4</sub>-2 were among the most active compounds for stimulating peritoneal macrophage lysosomal activity, while desulfation of their carbohydrate moiety completely abolished this activity [7]. Our study also indicated that the acetyl group at C-16' of the aglycon in frondoside A may play a significant role in frondoside's cytotoxicity and caspase activation since frondoside A had more potent effects than A<sub>4</sub>-2 on cytotoxicity, cell cycle changes and apoptosis but led to no caspase activation before early apoptosis. Frondoside A contains xylose in its carbohydrate residues, but the some changes in the carbohydrate residues may not play a significant role in the cytotoxicity of frondoside A and cucumariosides since the A<sub>2</sub>-2 and A<sub>4</sub>-2 differ only in the structure of their terminal monosaccharide residue (glucose and methylglucose, respectively). They show some differences in apoptotic activities but

similar effects on procaspase cleavage and mitochondrial permeability.

In the presence of frondoside A, annexin-V positivity preceded the loss of procaspases-8 and -3 after 6 h of treatment. Moreover, the annexin-V positivity was not inhibited by zVAD-fmk, whereas both the annexin-V positivity and cleavage of caspases induced by cucumariosides was efficiently blocked by caspase inhibitors. In addition, we observed inhibitory activity of zDEVD-fmk or zVAD-fmk in the frondoside A-induced cleavage of procaspases and PARP by Western blotting, indicating that the caspase inhibitors effectively blocked frondoside A-provoked activation of caspases. These results suggest that frondoside A caused caspase-independent cell death. Recently, Li et al. have shown that treatment of human pancreatic cancer cells with low concentration of frondoside A for 24 h or 48 h induces apoptosis of pancreatic cancer cells through the mitochondrial and activation of caspase cascade [13]. They also reported strong annexin V staining in the pancreatic cancer cells after frondoside A treatment for 3 h [13]. Caspase-3 is an effector



**Fig. 5.** Effects of caspase inhibitors on frondoside A- or cucumarioside-induced apoptosis of HL-60 cells. (A) HL-60 cells were pretreated with or without 200 μM zDEVD-fmk or 200 μM zVAD-fmk for 1 h and then challenged with frondoside A (1 μM) or etoposide (10 μM) for 6 h. Representative experiments are shown. (B) Cells were pretreated with 100 μM zDEVD-fmk or 50 μM zVAD-fmk for 1 h and further treated with 3 or 5 μM of A<sub>2</sub>-2 or A<sub>4</sub>-2 for 6 h. Results represent the mean ± S.D. of three independent experiments. (C) Cells were pretreated with caspase inhibitors as in panel A and further treated with 1 μM frondoside A or 5 μM A<sub>2</sub>-2 for 6 h. \*P < 0.05 (Student's t-test).

caspase and is involved in the later stages of cell death, but in our study, zDEVD-fmk and zVAD-fmk did not protect significantly against the apoptotic effects of frondoside A, and it only reduced cucumarioside-induced cell death. Although caspase activation is considered a hallmark of apoptotic cell death, other less-defined cell death pathways have been described that appear not to require caspase activation. Recently, mitochondria-mediated, caspase-independent apoptotic pathways were described [14]. The loss of mitochondrial transmembrane potential is generally mediated by opening of the mitochondrial permeability transition pores and as a consequence, the release of apoptogenic molecules such as apoptosis-inducing factor (AIF) or cytochrome c with subsequent caspase-independent or -dependent nuclear degradation [15]. In the present study, frondoside A- or cucumarioside-induced apoptosis occurred in the absence of mitochondrial transmembrane potential changes, the cytosolic accumulation of cytochrome c, and

caspase-9 activation, likely excluding the involvement of AIF and cytochrome c. In addition to mitochondria, other organelles, such as ER and lysosomes are targets of apoptotic initiation. Caspase-12 is proteolytically activated by upstream initiator caspase-7 following ER stress and calpain activation [16]. However, the cleavage of procaspase-7 was not observed after 2 h treatment with frondoside A, as there was no loss of procaspases-8 and -3 at the same time point. Moreover, frondoside A-induced apoptosis was not abrogated by the ER stress inhibitor, salubrinal (data not shown). Together, these results suggest that frondoside A induces apoptosis in HL-60 cells through a mechanism distinct from extrinsic and intrinsic apoptosis pathways.

In conclusion, the data presented in this study indicate that frondoside A and cucumariosides both possess antileukemic properties by inducing apoptosis. The mechanism of this action is uncertain and will be explored in future studies. Caspase-independent anti-leukemic agents, such as frondoside A will be useful to overcome chemoresistance such as occurs in acute myeloid leukemia, where resistance to chemotherapy is associated with defects in both the extrinsic and intrinsic pathways of apoptosis.

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